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**ESTUDO DO EFEITO DO ESTRESSE E DO FOTOPERÍODO SOBRE RITMOS DE  
TEMPERATURA CENTRAL E DE ATIVIDADE E REPOUSO**

Porto Alegre  
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Trabalho de conclusão de curso de graduação  
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“Tudo aquilo que o homem ignora não existe para ele. Por isso, o universo de cada um se resume ao tamanho do seu saber.”

**Albert Einstein**

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## 1 RESUMO

O estilo de vida moderno criou uma necessidade de ter-se produtividade cada vez maior, extrair-se o máximo possível do tempo disponível. Com isso, desenvolveram-se novas rotinas de trabalho, como *shift-work* (trabalho de turno), que permitem execução de tarefas em horários diversos devido ao advento da luz elétrica. Também aumentou-se o grau de cobrança, com cargas de trabalho muitas vezes tão grandes que precisam ser levadas para casa, ocupando parte do tempo de lazer já tornado escasso devido ao “terceiro turno” de tarefas domésticas. Porém, estudos já demonstraram efeitos patológicos destas rotinas. Foram estabelecidas conexões entre o afastamento dos indivíduos do padrão de iluminação natural e diversas doenças como síndromes metabólicas, infertilidade e transtornos mentais, além de levar a alterações na integridade dos ritmos biológicos, que são muito influenciados pela luz. O estresse crônico, por sua vez, também apresenta influência tanto sobre o sistema circadiano – que sincroniza os ritmos biológicos com periodicidade de cerca de um dia – quanto sobre processos de saúde-doença física e mental.

Modelos animais foram desenvolvidos para estudar os mecanismos envolvidos na relação entre exposição a padrões de luz-escuridão diferentes, ou exposição a situações estressantes, e alterações nos ritmos circadianos e nos processos fisiológicos. Porém, até hoje não há descritos na literatura modelos que associem mudanças de iluminação e estresse em um mesmo protocolo, o que representaria uma mimetização mais condizente com o que experimentamos em nosso dia-a-dia. Nesse sentido, o objetivo deste trabalho é estabelecer um modelo experimental para estudar o impacto da combinação de manipulação do fotoperíodo a estresse crônico sobre os ritmos biológicos, principalmente de atividade-reposo e temperatura central.

Camundongos BALB/c foram randomizados em 4 grupos experimentais: CT (Controle), CMS (*Chronic Mild Stress*, Estresse Crônico Moderado), PP (*Photoperiod*, Fotoperíodo) e PP+CMS (Fotoperíodo + Estresse Crônico Moderado). Os grupos CT e CMS permaneceram sob 12:12h claro-escuro (CE) ao longo de todo o experimento. Os camundongos do grupo CT não passaram por nenhuma

intervenção, enquanto os do grupo CMS foram submetidos ao protocolo de estresse crônico. Os grupos PP e PP+CMS passaram por 4 ciclos de 10:10h CE (retornando a 12:12h CE ao final da manipulação do fotoperíodo), após os quais o grupo PP não foi mais perturbado e o grupo PP+CMS foi submetido ao protocolo de estresse. Todos os animais tiveram seus ritmos de temperatura central e atividade-reposu registrados durante todo o experimento e os parâmetros acrofase, amplitude e %VE foram calculados e comparados entre grupos e fases do experimento.

Encontramos um atraso de fase nas acrofases de ambos os ritmos analisados durante o período de alteração do ciclo claro-escuro, com uma redução na amplitude do ritmo de temperatura. Também observamos um adiantamento de fase em ambas acrofases acompanhado de menor %VE durante a aplicação do protocolo de estresse crônico. O grupo PP+CMS, que passou por ambas as intervenções, foi o mais afetado, perdendo inclusive a robustez de seu ritmo de atividade durante o estresse crônico. Todos os parâmetros dos 4 grupos retornaram aos níveis basais após o término das intervenções, demonstrando a capacidade de recuperação do sistema circadiano.

O modelo foi estabelecido com sucesso, evidenciando alterações nos ritmos de atividade e temperatura semelhantes às descritas na literatura quando do uso de cada intervenção isoladamente e demonstrando que os ritmos são afetados de forma mais pronunciada quando os protocolos são utilizados de forma conjunta. Estes resultados salientam a importância de estudar-se mais profundamente como esses fatores, alterando o funcionamento do relógio biológico, podem causar outras mudanças fisiológicas e distúrbios de comportamento e, potencialmente, levar ao desenvolvimento de transtornos mentais.

## 2 INTRODUÇÃO

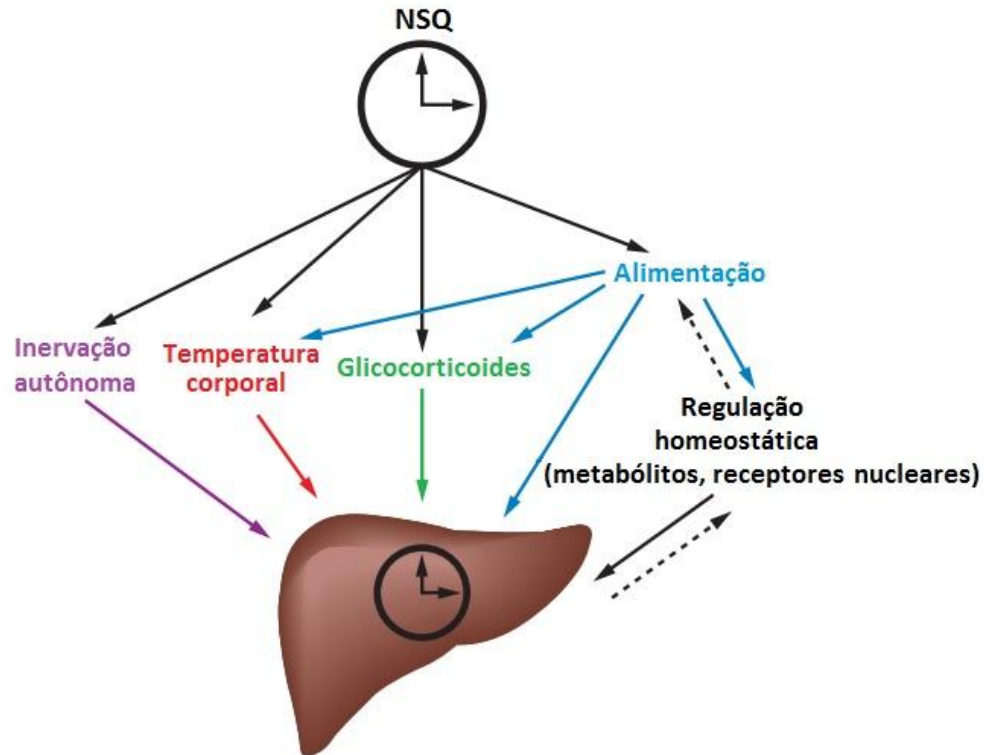
### 2.1 Cronobiologia e relógio biológico

A cronobiologia é uma área de conhecimento em expansão que vem ganhando popularidade desde que os avanços tecnológicos e as mudanças no perfil da sociedade tornaram as implicações patológicas dos estudos cronobiológicos mais evidentes (Roenneberg, 2013). Esta ciência, que investiga o papel do tempo sobre a vida (*kronos*, tempo; *bios*, vida; *logos*, estudo), visa compreender como a relação entre o organismo e o tempo – seja em escala de horários do dia ou mesmo de épocas do ano – se dá em situações de saúde e de doença, o que pode alterá-la e como é possível corrigi-la (Garaulet e Gómez-Abellán, 2013).

Um dos meios mais usados para aferição do aspecto cronobiológico é o monitoramento dos ritmos biológicos. Os ritmos circadianos – assim chamados por apresentarem ritmicidade de cerca de um dia – são regulados pelo sincronizador mestre de nosso relógio interno, o Núcleo Supraquiasmático (NSQ) do hipotálamo anterior. Esta estrutura é o principal responsável por interpretar as “pistas” do ambiente e indicar ao organismo a periodicidade de 24 horas do dia através de regulação de expressão gênica (Schibler e Sassone-Corsi, 2002). As células do NSQ apresentam individualmente *loops* de retroalimentação que regulam o processo de transcrição e tradução dos cerca de 20 genes envolvidos no sistema circadiano, chamados de *clock genes*, como *Bmal1*, *Clock*, *Period 1-3* (*Per 1-3*), *Cryptochrome 1/2* (*Cry 1/2*) e receptores nucleares. As proteínas CLOCK e BMAL1 formam um heterodímero que ativa a síntese de outras proteínas do sistema circadiano, como PERs e CRYs que, por sua vez, formam heterodímeros que reprimem a expressão da proteína BMAL1, formando um *loop* regulatório (Reppert e Weaver, 2002).

A sincronização central do NSQ gera sincronização de osciladores periféricos via inervação autônoma, controle da temperatura corporal, sinais humorais e pistas alimentares e, assim, estabelece ritmicidade dentro das aproximadamente 24h do dia na função de células e tecidos (Figura 1). Esses osciladores periféricos encontram-se em diversos tipos celulares fora do NSQ – como fígado, pâncreas, músculo esquelético, intestino e tecido adiposo – e auxiliam no controle circadiano

de processos como detoxificação xenobiótica, metabolismo, homeostase de glicose e lipogênese, por exemplo (Mohawk, Green e Takahashi, 2012).



**Figura 1:** Vias de sincronização de osciladores periféricos. O sincronizador central no núcleo supraquiasmático (NSQ) regula osciladores periféricos através de inervação autônoma, do controle da temperatura corporal, de sinais humorais (como glicocorticoides) e da alimentação. Adaptado de (Mohawk, Green e Takahashi, 2012).

## 2.2 Rimos circadianos

### 2.2.1 Hormônios e ciclo sono-vigília

Dentre os processos sob controle circadiano encontram-se neurotransmissão, metabolismo, imunidade e sinalização endócrina, como da glândula pineal, da hipófise e da adrenal. A pineal é uma glândula localizada no encéfalo com aproximadamente 5 mm de comprimento, 1-4 mm de espessura e com peso de cerca de 100mg que recebe aferências do núcleo paraventricular (NPV) do hipotálamo. Esta glândula possui dois tipos celulares: células neurogliais e, predominantemente, pinealócitos, cujo principal produto é o hormônio melatonina (Wu e Swaab, 2004). A melatonina é sintetizada em períodos de escuridão e



suprimida na presença de luz – ritmo regulado pelos sinais enviados do NSQ para o PVN e deste, para a pineal –, sendo o início de sua produção ao entardecer o marcador biológico de periodicidade circadiana mais confiável (Cajochen, Kräuchi e Wirz-Justice, 2003). Esta molécula exerce efeito sobre aspectos fisiológicos e comportamentais, destacando-se sua atividade sobre o ciclo sono-vigília em humanos, cujo ritmo é influenciado pela ação alternada de elevados níveis de melatonina produzidos durante a noite e reduzidos durante o dia. A administração de melatonina já é utilizada como tratamento de insônia, aumentando o tempo total de sono, melhorando a manutenção do sono e levando a uma maior frequência de fusos do sono em eletroencefalogramas (EEGs) (Kim, Jeong e Hong, 2015; Turek e Gillette, 2004).

O padrão de sono também sofre influência do eixo hipotálamo-hipófise-adrenal (HPA, de hipotálamo-pituitária-adrenal) via hormônio cortisol (ou corticosterona, em roedores) que, ao contrário da melatonina em humanos, tem seu pico no início da fase ativa e seus menores níveis do início da fase de inatividade. O sistema límbico, responsável pelas emoções e resposta ao estresse, sinaliza ao NPV do hipotálamo (também regulado pelo NSQ) que, em reação, envia hormônio liberador de corticotrofina (CRH, *corticotropin-releasing hormone*) para a hipófise anterior, induzindo liberação de hormônio adrenocorticotrófico (ACTH, *adrenocorticotrophic hormone*). O ACTH, então, estimula a síntese e liberação rítmica de glicocorticoides na corrente sanguínea, promovendo estado de alerta, mobilização de glicose e ácidos graxos e formação de memória na fase ativa do dia (Landgraf, McCarthy e Welsh, 2014).

### **2.2.2 Atividade-reposo e temperatura central**

Além do ciclo sono-vigília, muito avaliado através de polissonografia e EEG, outros dois ritmos circadianos são alvo de grande interesse em estudos cronobiológicos, principalmente em experimentação animal: atividade-reposo e temperatura central. Enquanto o ritmo de atividade-reposo é mais maleável, oscilando em um período de mais de 30 horas em situações de isolamento temporal – onde os indivíduos são privados de pistas que indiquem o horário do dia –, o ritmo de temperatura corporal é um dos ritmos circadianos mais robustos, se mantendo em um período de aproximadamente 24 horas nas mesmas condições (Cambras *et*

*al.*, 2007). Portanto, alterações de fase entre estes ritmos e o ciclo claro-escuro – fenômeno chamado de cronodisrupção –, verificadas através de aferição contínua das variáveis atividade e temperatura, são um indicador de alterações fisiológicas importantes em resposta a algum fator deletério (Erren e Reiter, 2013).

Devido aos impedimentos para realização de determinados estudos com humanos, torna-se necessário o uso de modelos animais para experimentos que envolvem fatores de risco e etiológicos. Nos estudos de alterações de ritmos e seus efeitos sobre comportamento e fisiologia, são utilizados principalmente modelos de roedores, apesar das diferenças interespecies de períodos endógenos, isto é, aqueles apresentados em isolamento temporal (Pilz *et al.*, 2015). Assim, diversos métodos já foram desenvolvidos para registro contínuo dos ritmos de atividade e temperatura em modelos animais, sendo os mais comuns: roda de atividade ou actígrafos – estruturas com feixes de laser que cruzam o interior das caixas-moradia dos animais e detectam seu movimento – para registro de atividade; sensores internos de temperatura, para registro de temperatura central (Pilz *et al.*, 2015); e telemetria, para registro e envio simultâneo via ondas de rádio dos dados de ambos os ritmos (Tokizawa *et al.*, 2015; Solarewicz *et al.*, 2015).

### 2.3 Fotoperíodo e sistema circadiano

Dentre os fatores que influenciam a ritmicidade do NSQ, o principal *zeitgeber* (do alemão, “doador do tempo”) é a luz. Tanto a luz natural como a luz artificial são percebidas por fotorreceptores contendo melanopsina presentes na retina dos mamíferos, que diferem dos demais receptores desta por não serem responsáveis pela formação de imagens, somente pela transdução de informações fóticas. O NSQ é informado da presença ou ausência de luz pelo trato retino-hipotalâmico e vias retinogeniculadas, originados das células ganglionares da retina, mas também recebe aferências dos núcleos da rafe, do mesencéfalo basal, da ponte, do bulbo e do hipotálamo posterior com informações não-fóticas (Dardente *et al.*, 2007).

Além de ser o *zeitgeber* mais importante, a luz também ocupa o papel de principal responsável por alterações circadianas. Em um estudo submetendo ratos jovens a avanços de fase crônicos, foram encontradas alterações metabólicas, inflamatórias e estresse do retículo endoplasmático. Esses resultados sugerem que rotinas que envolvem trabalhos de turno ou *jet lag* – cruzamento de fronteiras

meridionais que submete o indivíduo a um fuso-horário diferente – podem ter consequências deletérias (Herrero *et al.*, 2015). Pilz *et al.* (2014) demonstraram, também, que camundongos BALB/c submetidos a mudança no regime de luz de 12h-12h claro-escuro (CE) para 10h-10h CE sofrem alterações nos ritmos de atividade e de temperatura central, tendo suas amplitudes reduzidas após poucos ciclos de fotoperíodo alterado. Mesmo o ritmo de temperatura central, que é mais robusto, apesar de resistir inalterado por mais tempo, foi “achatado” pelo regime de luz modificado.

## 2.4 Ritmos vs. doença

Não somente a luz regula o funcionamento dos “relógios” centrais e periféricos. A alimentação, por exemplo, é um dos melhores sincronizadores não-fóticos, comparável ao efeito da luz sobre o sistema circadiano (Mistlberger, 2011). Isto é evidenciado em camundongos ou ratos, animais noturnos, que desenvolvem atividade antecipatória ao alimento (FAA, *food anticipatory activity*) se alimentados durante o dia. Os animais são capazes de lembrar o horário da alimentação através de “relógios internos” chamados de osciladores arrastados por alimento (FEO, *food entrainable oscillator*), exibindo FAA mesmo após vários dias de jejum (Tahara e Shibata, 2013). Esta relação entre a nutrição e o sistema circadiano faz com que alterações no sistema possam afetar o metabolismo. Camundongos com mutação no gene *Clock* apresentaram ritmo de alimentação enfraquecido e obesidade, independente do uso de dieta regular ou com alto teor de lipídeos (Turek *et al.*, 2005). Também camundongos duplo-*knockout* para *Per2* mostraram ritmo alimentar perturbado e obesidade com dieta rica em gorduras, causada por prejuízo nos ritmos de glicocorticoides (Yang *et al.*, 2009).

Além de estar associado a transtornos alimentares, o sistema circadiano está acoplado ao ciclo celular. A perda da ritmicidade circadiana por fatores externos ou por alterações moleculares dos *clock genes* pode afetar a proliferação celular e levar à geração de tumores, já que genes como *p53* (supressor de tumor), caspases (apoptose), ciclinas (ciclo celular), *c-Myc* (proto-oncogene) e *MDM2* (ubiquitina ligada a E3 que inibe *p53*) são alvos de elementos do relógio biológico (Fu e Lee, 2003). Estudos já demonstraram que inativação do gene *Per2* causa desregulação da expressão de *Bmal1* e, conseqüentemente, leva a uma superexpressão de *c-Myc* e ao desenvolvimento de tumores em camundongos (Fu *et al.*, 2002). Mudanças no

ciclo claro-escuro (8h de avanço do ciclo a cada 2 dias) também levaram a uma taxa de crescimento de tumores implantados em camundongos maior do que a de camundongos mantidos sob ciclo de 12h-12h CE, evidenciando o potencial nocivo de submeter-se a rotinas de alterações repetidas de fotoperíodo, como viajantes que passam por *jet lag* crônico (Filipski *et al.*, 2004).

## 2.5 Transtornos de humor e sistema circadiano

A teoria do *zeitgeber* social, por sua vez, implica na existência de pistas externas não-fóticas relacionadas a padrões de relações interpessoais, demandas sociais ou tarefas que ajustam o relógio biológico ao ciclo circadiano (Ehlers *et al.*, 1988). Atividades diárias que apresentam frequência e horário de execução regulares contribuem para o estabelecimento de rotinas e ritmos sociais estáveis. Dessa forma, eventos marcantes na vida dos indivíduos que causem mudanças nessas rotinas podem prejudicar a sincronização dos ritmos fisiológicos e comportamentais ao relógio externo (Grandin *et al.*, 2006). Eventos negativos poderiam, então, desencadear condições mentais como esquizofrenia, transtorno bipolar, ansiedade e depressão maior de forma direta ou indireta, via perturbações circadianas como alterações no ritmo de sono-vigília (Luca *et al.*, 2013).

Alguns sintomas clínicos clássicos de transtornos de humor incluem variação diurna de humor, acordar nos primeiros horários da manhã e distúrbios do sono, indicando uma periodicidade que relaciona a depressão à função circadiana. Encontram-se alterações como avanço de fase, redução de amplitude e sincronização variável às pistas sociais em vários ritmos de pacientes depressivos, como temperatura central, cortisol e metabolismo de monoaminas. Porém, não é possível afirmar se essa ritmicidade alterada seria a causa ou uma consequência da desordem de humor (Wirz-Justice, 2003). Apenas se pode afirmar, no momento, haver uma relação entre depressão e sistema circadiano, evidenciada pelos efeitos antidepressivos de melatonina em modelos animais (Detanico *et al.*, 2009) e humanos (Srinivasan *et al.*, 2014), bem como pelo potencial preditivo de resposta ao antidepressivo nortriptilina dos níveis do metabólito 6-sulfatoximelatonina (Hidalgo *et al.*, 2011).

## 2.6 Estresse e sistema circadiano

Dentre os fatores potencialmente prejudiciais aos ritmos biológicos e com associação a transtornos de humor, voltamos nosso interesse ao estresse, definido como qualquer situação capaz de perturbar a homeostase fisiológica ou psicológica, presente cada vez mais no nosso dia-a-dia (Palumbo *et al.*, 2007). O estilo de vida atual da sociedade, com demandas das mais diversas naturezas sendo impostas ao mesmo tempo e constantemente, impõe uma condição de estresse crônico muitas vezes inevitável sobre os indivíduos (Haridas *et al.*, 2013). Estudos já demonstraram que exposição a estresse, agudo ou crônico, leva ao desenvolvimento de transtorno depressivo (Kessler *et al.*, 1997), com sintomas como flutuações de humor, irritabilidade, alterações no apetite e no peso, anedonia, distúrbios de sono, fadiga e tendências suicidas.

A amplitude e a forma dos ritmos são influenciadas não somente pelo sistema nervoso central (SNC) e podem ser modificadas por estressores que afetam os sub-osciladores presentes nos diversos tecidos do organismo, ou que exercem outros efeitos de mascaramento dos sinais do sistema circadiano. Sabe-se que os glicocorticoides são capazes de resetar a ritmicidade de sincronizadores periféricos – induzindo *Per1* e *Per2* através de elementos responsivos a glicocorticoides (GREs, *glucocorticoids response elements*) –, sem alterar a função do sincronizador central no NSQ (Bartlang *et al.*, 2015). Esse desbalanço dos sinais fisiológicos pela interferência do estresse pode, então, levar a graves consequências na saúde física e mental dos indivíduos (Meerlo *et al.*, 2002).

. Sabendo-se que a resposta de estresse e a manutenção dos ritmos circadianos utilizam o eixo HPA como via comum, e tendo-se evidenciado relação entre estresse e transtornos de humor, torna-se interessante estudar os possíveis efeitos do estresse crônico sobre o relógio biológico. Takahashi *et al.* (2013) demonstraram que um protocolo de estresse crônico com camundongos BALB/c é capaz de induzir alterações nos níveis e na fase de liberação de corticosterona e na expressão de *clock genes* no fígado, um dos “relógios” periféricos, apesar de não gerar diferenças na expressão desses genes no NSQ. Com base nessas evidências

e na escassez de trabalhos que avaliem o efeito do estresse crônico sobre os ritmos, bem como sua possível interação com mudanças negativas no padrão de ciclo claro-escuro, decidimos estudar o efeito deste sobre os ritmos de atividade e repouso e de temperatura central em modelo animal, além de avaliar se uma alteração prévia no fotoperíodo pode amplificar o risco para mudanças nesses ritmos.

### **3 JUSTIFICATIVA**

Associações entre alterações no ciclo claro-escuro, prejuízo da ritmicidade biológica e desenvolvimento de distúrbios fisiológicos e psicológicos têm sido demonstradas através de experimentação animal. Também as relações entre situações de estresse, processos de saúde-doença e perturbações do sistema circadiano têm sido investigadas via modelos animais. Porém, estes modelos verificam apenas os efeitos de cada fator separadamente, enquanto o que experimentamos vivendo na sociedade atual é uma sequência ou sobreposição desses fatores. Para simular isto com maior similaridade, seria necessário um modelo que apresentasse alteração de fotoperíodo, a qual nos submetemos quase diariamente, seguida de período de estresse crônico, situação pela qual passamos nos ambientes de trabalho ou em relações interpessoais.

Observando esta necessidade, desenvolvemos este trabalho para validar um modelo animal que combina manipulação de fotoperíodo e estresse crônico moderado, e identificar os efeitos desta combinação sobre os ritmos biológicos de atividade-reposo e temperatura central como indicador da integridade de funcionamento do sistema circadiano.

## **4 OBJETIVOS**

### **4.1 Objetivo geral**

Verificar se alteração no fotoperíodo amplifica o risco para mudanças de ritmo após estresse.

### **4.2 Objetivos específicos**

- Verificar o efeito do estresse na amplitude, acrofase e mesor dos ritmos de temperatura e de atividade;
- Verificar se o estresse induz desacoplamento entre os ritmos de temperatura e de atividade;
- Verificar se alteração no fotoperíodo amplifica o risco para mudança de amplitude, acrofase e mesor dos ritmos de temperatura e de atividade após estresse;
- Verificar se alteração no fotoperíodo amplifica o risco para desacoplamento entre os ritmos de temperatura e de atividade após estresse.



## 5 ARTIGO CIENTÍFICO

Os resultados obtidos experimentalmente neste trabalho foram organizados na forma de artigo científico, a ser submetido ao periódico “*Journal of Neuroscience Methods*”.

**ESTABLISHMENT OF A MODEL FOR STUDY OF THE EFFECT OF  
PHOTOPERIOD MANIPULATION AND CHRONIC MILD STRESS OVER  
TEMPERATURE AND ACTIVITY-REST RHYTHMS**

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## Abstract

**Background:** There are already animal models for studying the effect of either stress or photoperiod manipulation over circadian rhythms and their implications in health issues. However, there is a lack of a protocol that associates light/dark alterations to chronic stress, which would represent a more realistic model to our modern life style. This study aimed to establish a model for understanding how photoperiod alteration previous to chronic mild stress can influence the circadian system, more specifically activity and temperature rhythms.

**Methods:** BALB/c mice had temperature and activity rhythms recorded by iButtons and actigraphs, respectively. Mice were sorted into 4 groups: CT (control), CMS (Chronic Mild Stress), PP (Photoperiod) and PP+CMS. CT and CMS mice remained under 12:12h light-dark (LD) cycles. CT mice were kept undisturbed and CMS mice underwent a CMS protocol. PP and PP+CMS mice underwent 4 cycles of 10:10h LD, after which PP mice were kept undisturbed and PP+CMS mice underwent the stress protocol. Acrophase, amplitude and %VE of rhythms were calculated and compared within groups and stages.

**Results:** We observed a phase delay in rhythms during photoperiod manipulation and a phase advance under CMS. Reduction of temperature amplitude was seen under 10:10h LD and lower temperature %VE, under CMS. PP+CMS group was the most affected, losing activity rhythm robustness under CMS. All parameters returned to basal after interventions.

**Conclusion:** Alteration in photoperiod previous to CMS results in stronger circadian impairment than each intervention on its own, highlighting the importance of further studying health impacts of this association.

**Keywords:** Chronic Mild Stress, Photoperiod Alteration, Activity, Temperature, Animal Model, Circadian Rhythm.

## 1. Introduction

Over-demanding work routines, with exorbitant schedules and responsibilities, are becoming the standard scenario in laborer's life. Nowadays, it is common to hear complaints about excessive work loads that surpasses the working schedules and end up being dispatched at home. This represents a two-way problem, since it causes an elongation of periods indoors under artificial illumination and submits individuals to chronic stressing conditions both in their work places and at home.

With the development of more economical light-bulbs and the popularization of mobile phones and digital devices, modern society experiences a phenomenon called light-pollution – when surplus light is directed to where it is not needed and maintains urbanized cities illuminated even at night – and an extreme shortening of exposure to total darkness (Chepesiuk, 2009). Along with the illumination, the possibility of prolonging work through night generated new work schedules (like shift-work, for example), and the necessity of spending the day at work and the night doing household chores (called the “third-shift”) represents an inescapable stressing environment (Wang *et al.*, 2011).

The circadian system, which regulates physiological rhythms by synchronizing them to the 24h-period of the day, is tightly linked to modifications in the light/dark cycle (Bedont and Blackshaw, 2015). Due to the evidences of interactions between photoperiod alterations, changes in biological rhythms and health issues, arose a necessity of animal models for understanding the mechanisms underlying this relation. From this, models of prolonged light exposure (Kooijman *et al.*, 2015), shift-work (Guerrero-Vargas *et al.*, 2015) and jet-lag (Atkinson *et al.*, 2014) were developed, and the connection with metabolic syndromes (Gangwisch, 2014), cancer (Kennaway, 2014), affective disorders (Bauer *et al.*, 2012) and other conditions was confirmed.

Affective disorders, mainly major depression, are also related to social and psychological consequences of chronic stress. Experimental models of chronic mild stress (Logan *et al.*, 2015; Haridas *et al.*, 2013) and early life stress (Zhang *et al.*, 2013) showed relation between undergoing stressing periods and depression-like behavior in mice, as well as changes in corticosterone level (the “stress hormone”)

and gene expression in the nucleus accumbens (brain structure of the reward pathway). Also, correlation among stress and the biological clock have already been proved by alterations in circadian rhythms (Bartlang *et al.*, 2015; Logan *et al.*, 2015) and molecular components of the internal clock (Takahashi *et al.*, 2012).

Although models of photoperiod manipulation and models of chronic stress are already established, there is a lack of protocols for understanding the effect of a combination of both interventions. Since we are constantly subjected to alterations in the light/dark cycle, and stressing situations are likely to happen in our day-to-day life, studying the impact (and maybe sensitization) of changes in illumination previous to the onset of stressors is a health issue. Therefore, we developed a model to fulfill this necessity and demonstrated, in this study, that the conjunction of these adverse conditions results in enhanced rhythmic dysfunctions in mice.

## 2. Materials and Methods

### 2.1. Experimental animals

Experiments were performed with 46-day-old male BALB/C mice (n=20) obtained from Universidade Federal de Pelotas. Animals were individually housed with food and water *ad libitum* in transparent acrylic home-cages (Panlab Harvard Apparatus; 25 x 15 x 25cm) with zeolites, since the equipment for activity detection does not allow the use of wood shavings. They remained in a photoperiod station with four separate independent chambers containing digital electronic timers (model TT34, COEL) which allow different photoperiod programming under the same controlled temperature ( $22 \pm 2$  °C), humidity and noise exposure. Mice were kept under a light-dark (LD) cycle of 12:12 h (unless otherwise stated), being weighed weekly throughout the experiment.

The 20 animals were divided into 4 experimental groups:

- CT (Control), n = 5: subject neither to the change in photoperiod, nor to the stress protocol;
- CMS (Chronic Mild Stress), n = 5: didn't undergo the change in photoperiod, but was subjected to the stress protocol;
- PP (Photoperiod), n = 5: subject to the change in photoperiod, but not to the stress protocol;
- PP+CMS (Photoperiod + Chronic Mild Stress), n = 5: subject to both the change in photoperiod and the stress protocol;

Mice were kept in our animal facility (Unidade de Experimentação Animal, UEA) of the Hospital de Clínicas de Porto Alegre (HCPA), under standard conditions and in groups of 5 animals for one week before the beginning of the experiments. All procedures were carried out according to institutional policies on animal use in research. This study was approved by the Ethics Committee of the institution (#12-0313 GPPG/HCPA).

## 2.2. Photoperiod Manipulation

The photoperiod modification protocol used in this study was previously established by our research group and described by Pilz *et al.* (2014). The alteration in photoperiod was held after 10 days under a 12:12 h LD regime reserved for verification of activity and temperature rhythms. Animals of groups without the change in photoperiod (CT and CMS) were kept under cycles of 12:12 h LD. Animals of groups subjected to the modification (PP and PP+CMS) were submitted to 4 cycles of 10:10 h LD during approximately 3 days, after which they were returned to 12:12 h LD.

## 2.3. Chronic Stress Protocol

The chronic mild stress (CMS) protocol used was an adaptation of the methodology described by Haridas *et al.* (2013). After the stage of photoperiod manipulation, all animals were kept under 12:12 h LD cycles and those belonging to stressed group (CMS and PP+CMS) were exposed to the stress protocol for 21 days. Animals were subjected to three different randomized stressors every day, in 3 periods of the animals' subjective day indicated in zeitgeber times (ZT), being ZT 0 the moment the lights were turned on and ZT 12, when they were turned off. The stressors were administered at: first half (ZT 3,5 to ZT 5,5), second half (ZT 7,5 to ZT 9,5) and overnight (ZT 11,5 to ZT 2,5 of the following day). After the 21 days, mice were kept without stress for 10 days for verification of activity and temperature rhythms post-CMS.

Briefly, the stressors used were: Cold, in which mice were placed on a bedding of icepacks; Space Reduction, by placing a partition which limited the available space to 50%; Cage Tilt in 45°; Wet Bedding, by addition of 100 ml of water to the zeolites; No Bedding, by placing the animal in a home-cage without zeolites; Rat Bedding, using wood shavings from rats' home-cages; and Restraint Stress, performed with a perforated tube of 10 cm height and 3 cm diameter ending in a cone of 2 cm height. Table 1 shows the application order of the stressors over a week, which was repeated three times.

**Table 1.** Stress regime applied in each day of one week of chronic mild stress. This protocol was repeated for a total of three weeks.

	<b>1st half</b> (duration: 2h)	<b>2nd half</b> (duration: 2h)	<b>Overnight</b> (duration: 15h)
<b>Day 1</b>	Cold	Cage-Switch	Space Reduction
<b>Day 2</b>	Cage Tilt	Wet Bedding	No Bedding
<b>Day 3</b>	Space Reduction	Restraint Stress	Rat Bedding
<b>Day 4</b>	No Bedding	Space Reduction	Cage Tilt
<b>Day 5</b>	Cage-Switch	Cold	Space Reduction
<b>Day 6</b>	Wet Bedding	Cage Tilt	No Bedding
<b>Day 7</b>	Restraint Stress	No Bedding	Rat Bedding

## 2.4. Rhythm Characterization

*Rest-activity:* The activity rhythm of each animal was monitored daily every 10 minutes by actigraphy (ADNpln- © Antoni Díez Noguera, Barcelona, AC, Spain). This device emits two laser beams that cross the animal's home-cage longitudinally and transversely and are detected by receivers on the opposite face of the cage. When a beam is interrupted by the interposition of the animal when it moves, one locomotion unit is recorded. Each device is connected to a board responsible for converting its information to digital format and sending them to a computer, where the data can be accessed at DAS192USB program. The recordings of activity were converted into Excel files and organized according to the stages of the experiment: Pre-experimentation (Pre); Photoperiod manipulation (Photoperiod); CMS protocol (Stress); and Post-experimentation (Post). The data was then prepared for analysis using specific software to measure rhythmicity parameters.

*Core temperature:* The temperature rhythm of each animal was measured by internal temperature sensors (Thermochron iButtons, Dallas, TX) implanted after disinfection, into the peritoneal cavity. They were surgically implanted via an incision of approximately 2 cm under anesthesia with isoflurane (5% induction and maintenance of 2-3%) and using 4.0 Vycril thread as the internal suture and 4.0



Nylon thread as external suture. The animals were kept in incubators and under observation until recovery from surgery, and then were returned to their home-cages. On the day of surgery and on the two following days, animals received intraperitoneal injections of analgesic Tramadol (10mg / kg) every 12 hours. The sensors recorded the core body temperature of the animals every 45 minutes. The iButtons were recovered after euthanasia by decapitation and data was extracted through specific equipment and software (1-Wire® devices, Dallas, TX). The first week after surgery was considered recovery period and data from this period was disregarded. The recordings were converted to Excel, organized in the different experimental stages and prepared, as the activity data, for analysis in specific software to measure the rhythmicity parameters.

## **2.5. Statistical Analysis**

The evaluation of rhythms was performed using the integrated program of Chronobiology El Temps (A. Díez-Noguera, Universitat de Barcelona, 1999) to calculate rhythmicity parameters - amplitude, acrophase, mesor and VE% of the activity and temperature recordings - through cosinor analysis, as well as to run Rayleigh tests for acrophases and to generate actograms.

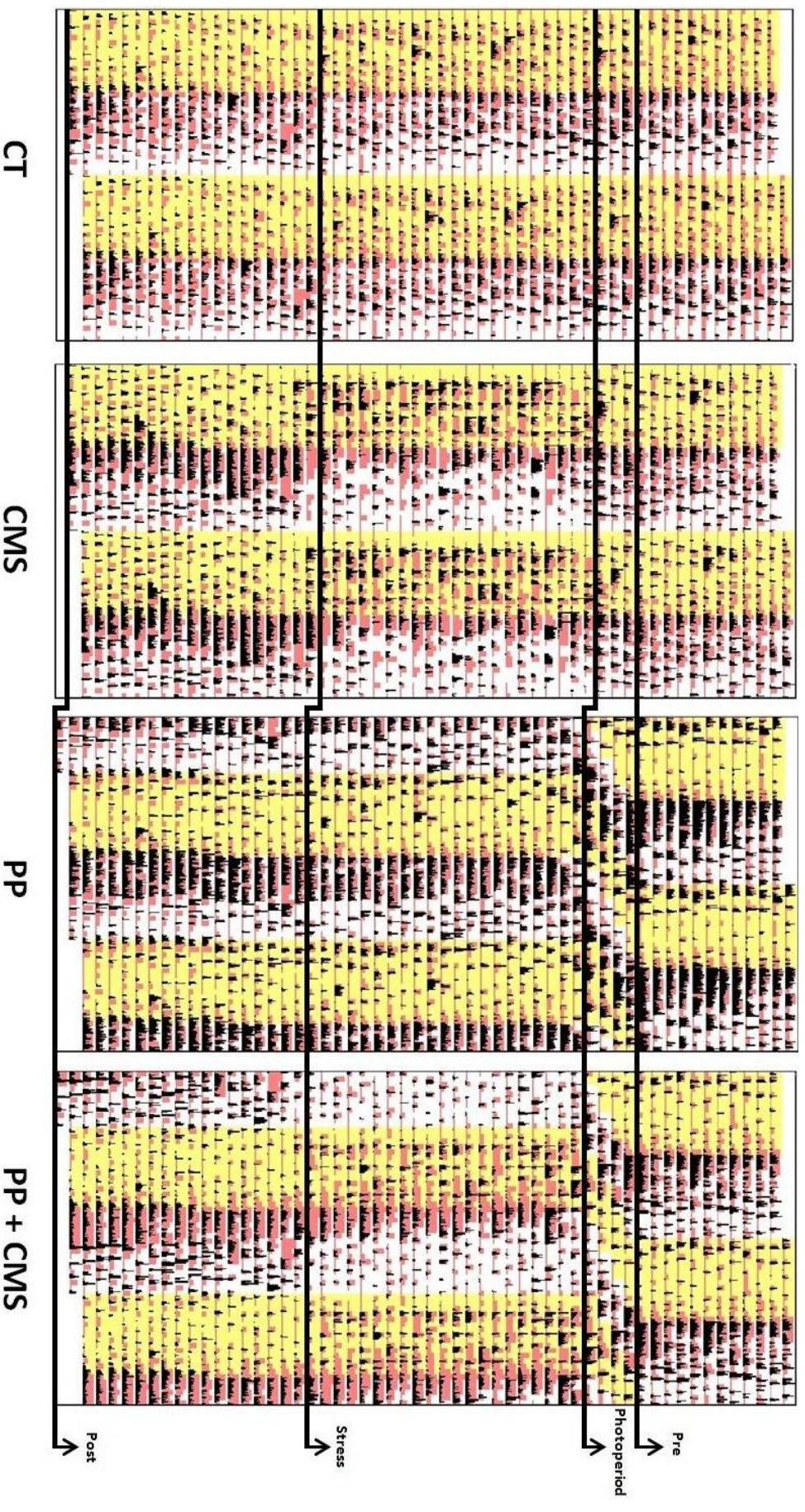
Comparison of the parameters between different stages within each experimental group was made using ANOVA for repeated measures followed by Tukey test for multiple comparisons. The analysis of differences between groups within each stage was performed by one-way ANOVA with Tukey post-hoc test. Data are presented as means  $\pm$  standard deviations (SD), with the statistical significance level set at  $p \leq 0.05$ . Graphics were generated in GraphPad Prism 6 software and analysis were performed using SPSS 22.0 software (SPSS Inc, Chicago, IL, USA).

### 3. Results

Aiming to identify the impact of CMS, photoperiod modification and the combination of both interventions over the circadian system, we evaluated the entrainment of activity and temperature rhythms to the light/dark cycles throughout the experiment, shown in Figure 1. The pattern of the rhythms under control conditions is seen in the CT group, with the peaks of activity and temperature occurring always in the first-half of the dark phase. When subjected to CMS, mice showed a phase advance in both activity and temperature to the light phase, but both rhythms returned to its basal form after the administration of the stress protocol. Mice in the PP group, that underwent the 10:10h LD period, presented a phase delay in the rhythms that reached its maximum at the last cycle of photoperiod modification, but showed reentrainment to the light/dark cycle through the following days. Lastly, the group PP+CMS exhibited the delay in both rhythms as the PP group during the photoperiod alteration, followed by a phase advance similar to the CMS group over the stress period and a reentrainment through the remaining days of experiment.

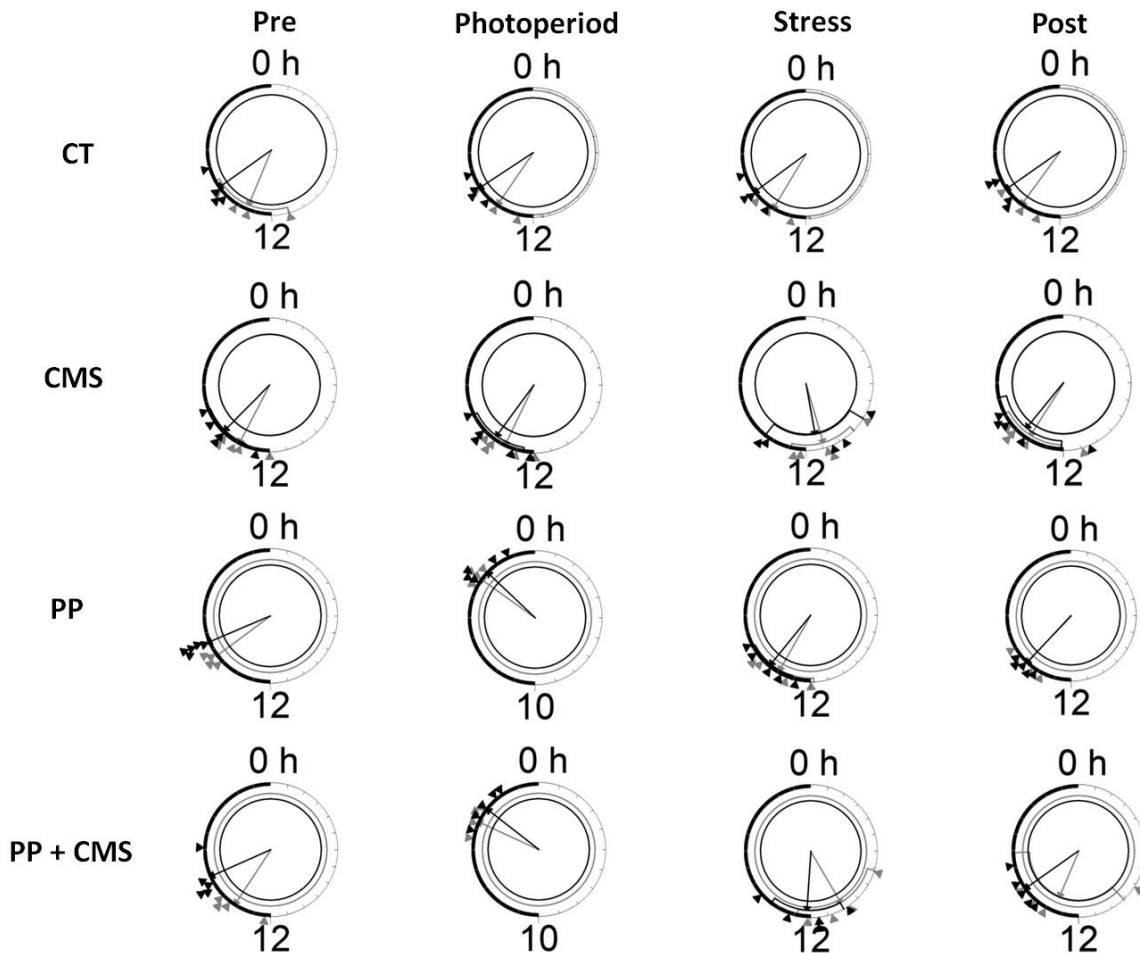
The acrophases of activity and temperature rhythms of each group in each stage of the experiment are shown in Figure 2. As seen in Figure 1, group CT acrophases of both rhythms remained in the first-half of the dark phase - activity around ZT 15 and temperature, ZT 13 - through the whole experiment in the absence of interventions. The groups PP and PP+CMS, which were subjected to the photoperiod manipulation, showed a delay in the acrophases of activity (around ZT 17 of the 20h-day) and temperature (close to ZT 17 in the PP group and to ZT 16 in the PP+CMS group) during the modification of the light/dark cycle. Over the application of the stress protocol, groups CMS and PP+CMS exhibited an advance in the acrophases, with both acrophases being around ZT 11 in CMS group and close to ZT 10 and ZT 12 for temperature and activity, respectively, in PP+CMS group. Acrophases of all groups returned to the first-half of the dark phase after the interventions.

Regarding the changes in other rhythmicity parameters through the experiment, we found that the temperature amplitude (Figure 3A) was significantly different depending on the stage it was recorded for all groups [CT:  $F_{(3, 9)} = 4,61$ ,  $p = 0,032$ ; CMS:  $F_{(3, 16)} = 6,71$ ,  $p = 0,004$ ; PP:  $F_{(3, 12)} = 16,39$ ,  $p = 0,000$ ; PP+CMS:  $F_{(3, 12)}$



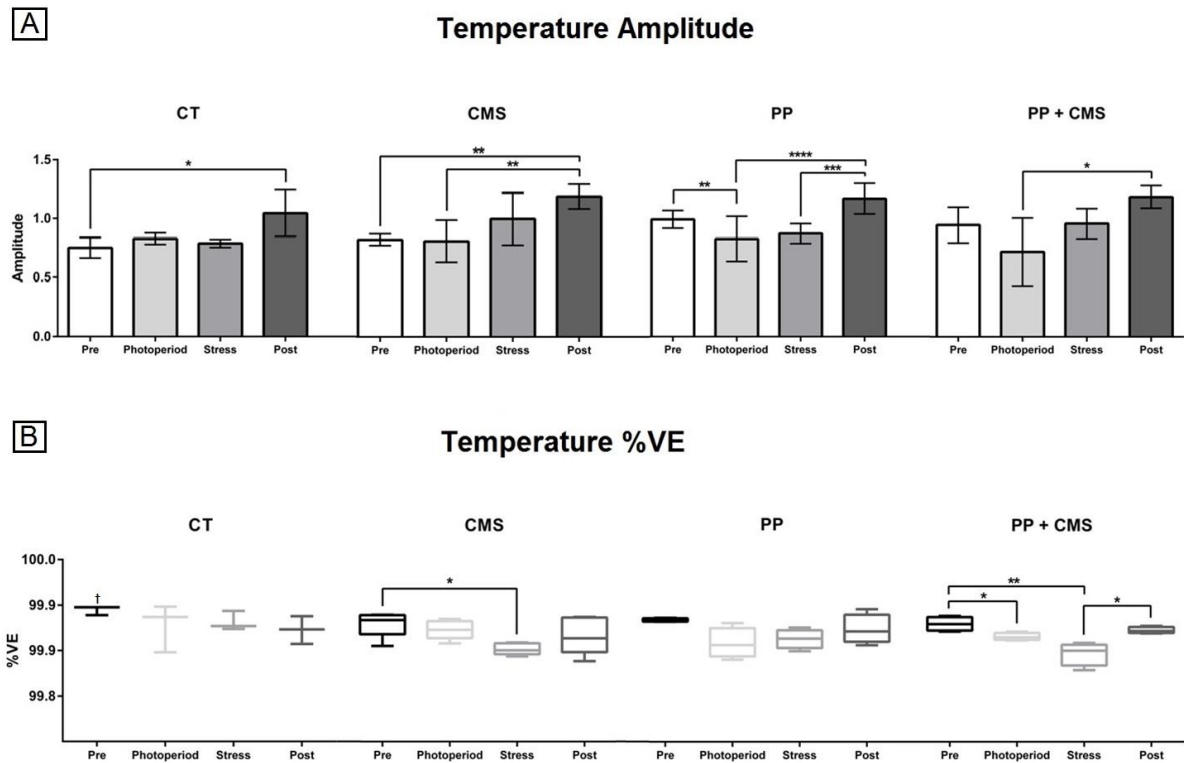
**Figure 1: Effect of photoperiod modification and/or stress in rest-activity and temperature rhythms.** Representative double-plot actograms from each group, depicting daily variance of rest-activity (black) and core body temperature (red) rhythms throughout the experiment. Lights-on periods are shown in yellow. The pattern shows phase advance of both rhythms during Photoperiod stage in the groups subjected to 10:10h LD cycles (PP and PP+CMS), phase delay during Stress stage in the groups that under the CMS protocol (CMS and PP+CMS), and reentrainment to the light/dark cycle in the Post stage. CT: Control; CMS: Chronic Mild Stress; PP: Photoperiod; PP+CMS: Photoperiod + Chronic Mild Stress.





**Figure 2: Photoperiod manipulation and CMS protocol change acrophases of activity and temperature rhythms.** Rayleigh tests of activity (black) and temperature (gray) acrophases, demonstrating the phase delay of acrophases under photoperiod modification, the phase advance under stress protocol and the recovery post-experimentation; 0 h represents the time when the lights were turned on.

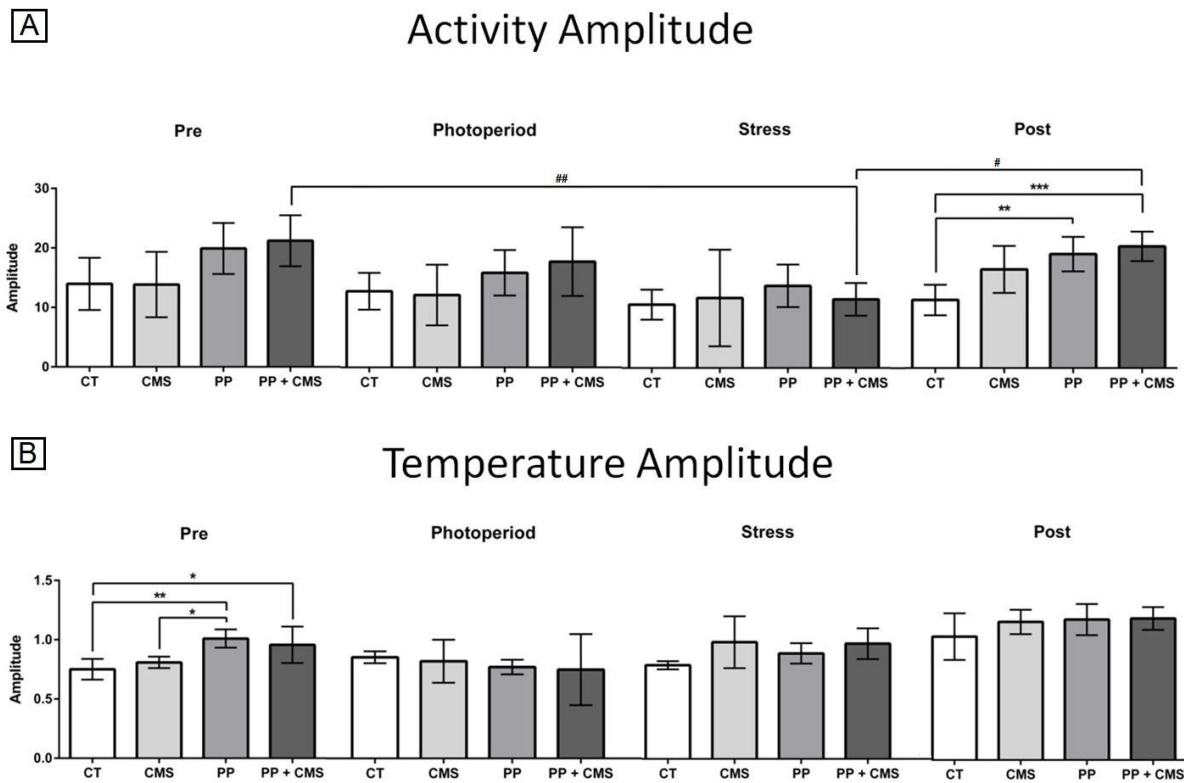
= 4,41,  $p = 0,026$ ]. The means of temperature amplitude in the Post stage ( $M = 1,04$ ,  $SD = 0,20$ ) were statistically higher than in the Pre stage ( $M = 0,75$ ,  $SD = 0,09$ ) for CT group. The Pre ( $M = 0,81$ ,  $SD = 0,05$ ) and Photoperiod ( $M = 0,80$ ,  $SD = 0,18$ ) stages, where no intervention was made, had lower temperature amplitudes than the Post stage ( $M = 1,17$ ,  $SD = 0,10$ ) for CMS group. For the PP group, the temperature amplitude at the Pre stage ( $M = 1,01$ ,  $SD = 0,08$ ) was higher than during the Photoperiod alteration ( $M = 0,75$ ,  $SD = 0,06$ ), and both Photoperiod and Stress ( $M = 0,89$ ,  $SD = 0,09$ ) stages had lower amplitudes than the Post stage ( $M = 1,19$ ,  $SD = 0,13$ ). In the PP+CMS group, the only significant difference found was an increase in the amplitude in the Post stage ( $M = 1,20$ ,  $SD = 0,10$ ) in comparison to the Photoperiod manipulation stage ( $M = 0,73$ ,  $SD = 0,19$ ).



**Figure 3: Impact of experiment stage over temperature amplitude and %VE within each group.** Differences in temperature amplitude (A) and %VE (B) in each stage within groups CT (n=3; Pre: n=4), CMS (n=5), PP (n=4) and PP+CMS (n=4). Amplitude and %VE presented as means  $\pm$  SD. \*  $p \leq 0,05$ ; \*\*  $p \leq 0,01$ ; \*\*\*  $p \leq 0,005$ ; \*\*\*\*  $p \leq 0,001$ , ANOVA/Tukey. † outlier removed (%VE = 99,5819).

We also found an effect of the experiment stage in the temperature %VE (Figure 3B), which indicates how much the variable follows a circadian rhythmicity, only in groups subjected to the stress protocol: CMS and PP+CMS [CMS:  $F_{(3, 16)} = 3,74$ ,  $p = 0,033$ ; PP+CMS:  $F_{(3, 12)} = 7,38$ ,  $p = 0,005$ ]. CMS group showed a reduction in temperature %VE from Pre stage (M = 99,93, SD = 0,01) to Stress stage (M = 99,90, SD = 0,01). PP+CMS group exhibited lower %VE in Photoperiod stage (M = 99,91, SD = 0,02) and, more markedly, in Stress stage (M = 99,90, SD = 0,01) than in the Pre stage (M = 99,93, SD = 0,01). At the Post stage (M = 99,92, SD = 0,00), %VE elevated to a significantly higher value than at the Stress stage, being similar to the Pre stage %VE.

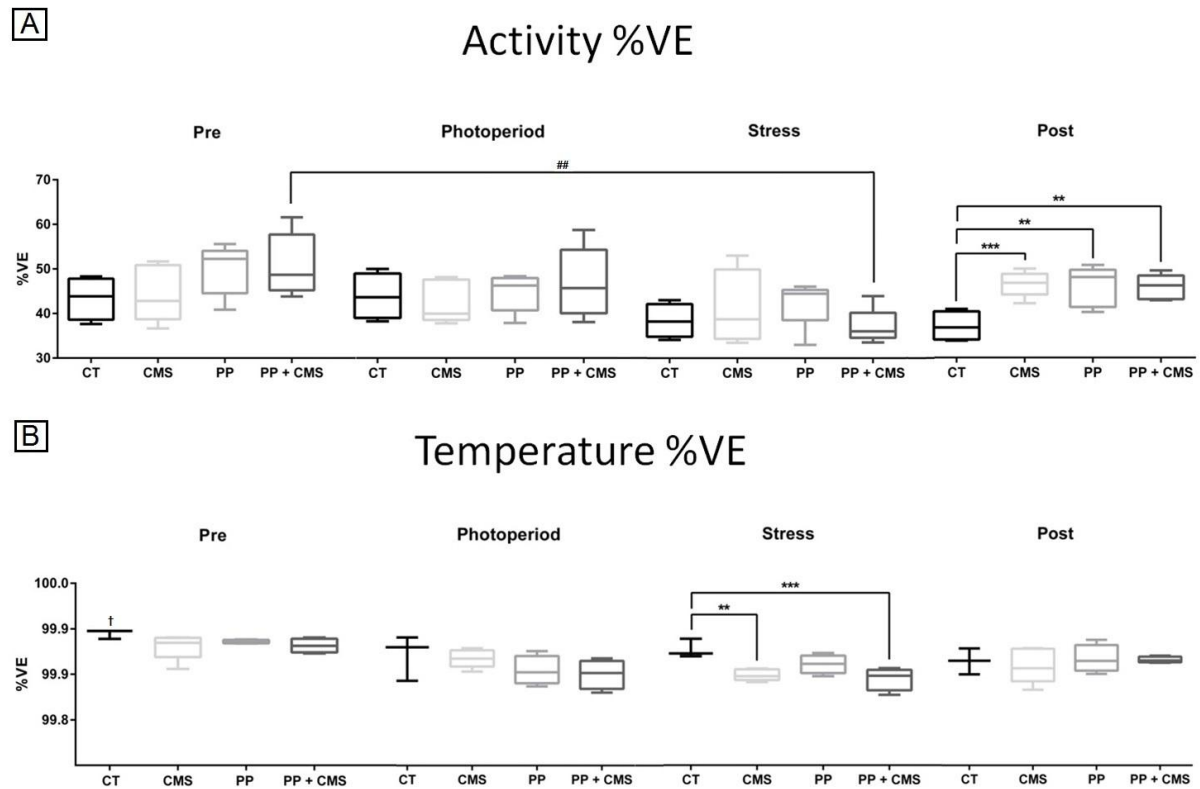
Next, we compared the amplitudes of activity and temperature between groups in each stage. Results are presented in Figure 4. In temperature amplitude (Figure 4B), the only difference found was between groups in the Pre stage [ $F_{(3, 13)} = 6,60$ ,  $p = 0,006$ ], when the CT amplitude (M = 0,75, SD = 0,09) was significantly lower than that of PP (M = 1,01, SD = 0,08) and of PP+CMS (M = 0,96, SD = 0,15),



**Figure 4: Differences in activity and temperature rhythms amplitudes between groups in each stage.** Differences between activity amplitude (A) and temperature amplitude (B) of each group within each stage. Activity amplitude: CT (n=4), CMS (n=5), PP (n=5) and PP+CMS (n=5). Temperature amplitude: CT (n=3; Pre: n=4), CMS (n=5), PP (n=4) and PP+CMS (n=4). Data presented as means  $\pm$  SD. \*  $p \leq 0,05$ ; \*\*  $p \leq 0,01$ ; \*\*\*  $p \leq 0,005$ ; \*\*\*\*  $p \leq 0,001$  comparing groups within stages; #  $p \leq 0,05$ ; ##  $p \leq 0,01$  comparing group PP+CMS between stages, ANOVA/Tukey.

and CMS amplitude ( $M = 0,81$ ,  $SD = 0,05$ ) was also lower than that of PP. In activity amplitude (Figure 4A), there were differences between groups in the Post stage [ $F_{(3, 15)} = 7,38$ ,  $p = 0,003$ ], when both PP ( $M = 18,56$ ,  $SD = 2,81$ ) and PP+CMS amplitudes ( $M = 19,82$ ,  $SD = 2,40$ ) were higher than CT value ( $M = 11,08$ ,  $SD = 2,48$ ).

In Figure 5, the results of the analysis of activity and temperature %VE between groups within stages are shown. Differences in activity %VE (Figure 5A) were found only in the Post stage [ $F_{(3, 15)} = 7,44$ ,  $p = 0,003$ ], when the CT group ( $M = 36,94$ ,  $SD = 3,26$ ) had lower %VE than the other three groups (CMS:  $M = 46,23$ ,  $SD = 2,77$ ; PP:  $M = 45,75$ ,  $SD = 4,39$ ; PP+CMS:  $M = 45,58$ ,  $SD = 2,73$ ). In the Stress stage, significant changes were observed in temperature %VE [ $F_{(3, 12)} = 7,41$ ,  $p = 0,005$ ] (Figure 5B) between groups CT ( $M = 99,93$ ,  $SD = 0,01$ ) and the groups that underwent the chronic stress protocol (CMS:  $M = 99,90$ ,  $SD = 0,01$ ; PP+CMS:  $M = 99,90$ ,  $SD = 0,01$ ).



**Figure 5: Differences in activity and temperature rhythms %VE between groups in each stage.** Differences between activity %VE (A) and temperature %VE (B) of each group within each stage. Activity %VE: CT (n=4), CMS (n=5), PP (n=5) and PP+CMS (n=5). Temperature %VE: CT (n=3; Pre: n=4), CMS (n=5), PP (n=4) and PP+CMS (n=4). Data presented as means  $\pm$  SD. \*  $p \leq 0,05$ ; \*\*  $p \leq 0,01$ ; \*\*\*  $p \leq 0,005$ ; \*\*\*\*  $p \leq 0,001$  comparing groups within stages; ##  $p \leq 0,01$  comparing group PP+CMS between stages, ANOVA/Tukey. † outlier removed (%VE = 99,5819).

Besides the comparison within stages, we observed a significant impact of the stage within the PP+CMS group regarding to the activity amplitude [ $F_{(3, 16)} = 5,96$ ,  $p = 0,006$ ] (Figure 4A) and %VE [ $F_{(3, 16)} = 5,11$ ,  $p = 0,011$ ] (Figure 5A). In this group, the amplitude reduced from the Pre stage (M = 21,21, SD = 4,29) to the Stress stage (M = 11,34, SD = 2,70), and recovered to higher values at the Post stage (M = 19,82, SD = 2,40). Similarly, the %VE was lower in the Stress stage (M = 37,02, SD = 3,92) than in the Pre stage (M = 50,90, SD = 7,00), but the recovery was not as prominent as in the amplitude.

#### 4. Discussion

In this study, we validated a model of chronic mild stress protocol, photoperiod manipulation and the combination of these interventions, showing that each of these factor have different effects over biological rhythms of temperature and activity-rest of male BALB/c mice. Some of the significant differences found, however, are not consequence of the protocols administered. For example, the only explanation we could think for the differences in temperature amplitude seen in the Pre stage is variability between the natural physiology of the mice in each group. The elevated temperature amplitude seen in the Post stage comparing to other stages in all groups is probably due to the aging of the animals, since they began the experiment 46-days old and, at the Post stage, they had between 86 and 104 days. Also, differences between activity amplitude of PP and PP+CMS groups and CT group in the Post stage are probably due to the basal higher activity amplitude in the Pre stage summed to an exacerbation of this amplitude in the recovery from the interventions these groups underwent.

Pilz *et al.* (2014) showed that a protocol of 10 days (12 cycles) of shortened photoperiod from 12:12h LD to 10:10h LD led to delayed temperature acrophase, reduced temperature amplitude and period alteration of this rhythm in BALB/c mice, suggesting that this strain is more susceptible to photoperiod manipulations. Here we subjected the animals to 4 cycles of 10:10h LD, based on the results of Pilz *et al.* (2015) that demonstrated a maximum disruption of activity and temperature rhythms after only 4 cycles of shortened photoperiod, after which mice proceeded with attempts to resynchronize their rhythms to the altered LD cycle. As expected, we found a delay in acrophases of both rhythms under photoperiod alteration to 10:10h LD, as well as a decrease in temperature amplitude (group PP) and %VE (group PP+CMS). Differently from Pilz *et al.*, we did not find significant differences in activity amplitude, however this must be due to our small number of animals per group ( $n = 5$  at the beginning of the experiment) and to the high variability in some measures, evident through the large standard deviations.

We also found a phase advance in acrophases of activity and temperature rhythms over the application of the stress protocol, with reduction of the circadian variability component (%VE) of temperature rhythm. Bartlang *et al.* (2015)



demonstrated that C57BL/6J mice exposed to repeated social defeat stress had an increase in activity and body temperature during each stress session, followed by a decrease in activity over the remaining day, independent of the time of stress exposure. A similar effect can be seen in our results, since BALB/c mice exhibited elevated temperature and activity during the CMS protocol over the light phase, when 2 of the 3 daily stressors were administered, and this elevation was compensated by a decreased activity through the dark phase. This is also corroborated by our findings of advanced acrophases, aspect not evaluated by Bartlang *et al.*, that indicate a shift of the rhythms peaks to the light phase.

Regarding to the group PP+CMS, we found that manipulation of photoperiod, which delayed rhythms acrophases, previous to application of the CMS protocol resulted in a magnified effect in the advance of temperature acrophase and a not so effective advance in activity acrophase. We observed a reduction in temperature %VE in both Photoperiod and Stress stages, with the difference between Pre stage and Stress stage being stronger than observed in the CMS group, but still capable of returning to basal values in the Post stage. Also, we found that activity amplitude of PP+CMS group decreased significantly from Pre stage to Stress stage, which couldn't be observed in the groups subjected to only one of the interventions. This highlights the potentiated effect of altering the photoperiod previously to implementing the stress protocol over the alterations in biological rhythms.

The association between chronic stress and the circadian clock system has been studied, either by evaluation of circadian rhythms (Gorka, Moryl and Papp, 1996; Thompson *et al.*, 2014) or expression of clock genes (Takahashi *et al.*, 2013), as well as the connection between photoperiod alterations and the biological clock (Oishi *et al.*, 2015). However, these works only investigate each intervention separately, disregarding its combined outcome. Besides its effects over the circadian system, changes in photoperiod are associated with affective disorders such as major depression (Green *et al.*, 2015) and bipolar disorder (Young *et al.*, 2015), conditions that can arise from periods of chronic stressing conditions (Czéh *et al.*, 2015) and are linked to rhythmicity perturbation (Landgraf *et al.*, 2014). This underscores the importance of our work for further understanding the mechanisms involved in the association of light/dark alterations (like shift-work or jet lag) and stress (such as

post-traumatic stress disorder), to later cultivate actions for preventing the onset of mental conditions.

In this study, we developed a model to investigate the impact of photic manipulation and/or stress over circadian rhythms. We demonstrated that acute photoperiod alterations previous to chronic mild stress periods sensitize animals to reductions in activity and temperature rhythms robustness, what could lead to behavioral disturbances and, possibly, to development of mental disorders. Therefore, these findings shed light over the potential hazard of subjecting individuals (or oneself) to unnatural light/dark regimes that could be followed or accompanied by stressing situations, a likely scenario in the modern society.

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## 6 CONCLUSÕES E PERSPECTIVAS

Neste trabalho, estabelecemos um modelo animal que permite avaliar os efeitos de manipulação do fotoperíodo e de estresse crônico moderado, independentemente e em conjunto. Pudemos identificar a influência destas intervenções sobre os ritmos de temperatura central e atividade-reposo, observando resultados condizentes com a literatura quando da aplicação de cada intervenção separadamente: atraso da acrofase e redução da amplitude da temperatura sob regime de 10:10h CE; e adiantamento da acrofase e redução do componente circadiano (%VE) da temperatura sob protocolo de estresse crônico. Além de confirmarmos a validade de nosso modelo através dos resultados corroborados por outros estudos, demonstramos que a combinação de manipulação de fotoperíodo com estresse resulta em efeitos mais pronunciados sobre os ritmos biológicos avaliados, e que todos os grupos experimentais foram capazes de recuperar o padrão rítmico basal após suspendermos as intervenções.

Tendo determinado o efeito sobre os parâmetros rítmicos avaliados neste primeiro estudo, pretendemos aumentar o tamanho amostral – reduzido neste primeiro momento por questões técnicas de espaço e tempo reduzidos – para tentar identificar outras alterações que possam não ter sido estatisticamente significativas devido ao número limitado de animais. Além disso, iremos proceder a análises de variabilidade interna dos ritmos avaliados para identificar possíveis mudanças não identificadas pelos métodos de análise utilizados.

Quando procedemos à eutanásia, amostras de soro foram coletadas e os encéfalos dos animais foram removidos e congelados. Iremos analisar, nas amostras de sangue, as concentrações de corticosterona e de melatonina, para comparar o estresse (via corticosterona) e o estado do relógio biológico (via melatonina) ao final do experimento, quando já observamos uma recuperação dos ritmos avaliados ao padrão basal que pode não se refletir no nível bioquímico. Os encéfalos serão analisados por técnicas de proteômica para verificação de mudanças em proteínas e receptores que podem estar envolvidos nas alterações que encontramos no sistema circadiano.

Por fim, realizaremos testes comportamentais no modelo desenvolvido para estudar a influência da combinação da manipulação de fotoperíodo com estresse crônico moderado sobre questões psicológicas não relativas aos ritmos. Através do teste claro-escuro, avaliaremos a expressão de ansiedade dos animais e, com o teste de preferência por solução de sacarose (adaptado para padronização durante a realização deste trabalho), identificaremos a presença de comportamento tipo-depressivo. Desta forma, poderemos determinar se a conjunção das intervenções representa um risco potencial de desenvolvimento de transtornos de humor maior do que cada fator individualmente.



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# JOURNAL OF NEUROSCIENCE METHODS

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*The Journal of Neuroscience Methods* publishes papers that describe new methods that are specifically for neuroscience research conducted in invertebrates, vertebrates or in man. Major methodological improvements or important refinements of established neuroscience methods are also considered for publication. The Journal's Scope includes all aspects of contemporary neuroscience research, including anatomical, behavioural, biochemical, cellular, computational, molecular, invasive and non-invasive imaging, optogenetic, and physiological research investigations.

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